Applied StemCell, Inc.
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Datasheet

TARGATT™ HEK293 Master Cell Line & Knockin Kit

Product Information

Catalog Number **AST-1305**

Description

The TARGATT™ HEK293 Master Cell Line and transgenic kit was designed for fast and site-specific knockin in HEK293 cells, using an easy-to-use gene knockin approach. The master cell line provided in this kit contains an "attP" integrase-recognition landing pad engineered into the hH11 safe harbor locus in the genome. The kit also contains a cloning plasmid containing a corresponding "attB" sequence into which any gene of interest can be cloned (under control of the CMV promoter or a promoter-of-choice). The expression of the integrase (provided as an integrase plasmid) mediates the stable integration of the transgene into the master cell line (Figure 1). The TARGATT™ integrase technology enables highly efficient (>40% without enrichment and ~90% with enrichment), and site-specific DNA integration without disruption of internal genes. The TARGATT™ HEK293 master cell line and knockin kit is ideal for single transgene gene knockin and uniform, stable expression of your protein*.

The TARGATT™ HEK293 master cell line and knockin kit are suitable for research applications involving directed-evolution of proteins (vaccine development, drug screening, cell-based gene therapy), genome-wide screening, and other stable cell line generation applications**.

**The TARGATT™ HEK293 Master Cell Line is also suitable for building mammalian cell libraries. However, the included TARGATT™ cloning plasmid is not suitable for generating the TARGATT™ mammalian cell libraries. If your application involves cell library preparation, please contact Applied StemCell for details about our TARGATT™ library cloning plasmids.*

***TARGATT™ master cell lines can be generated in any cell line including stem cells. Please contact Applied StemCell for TARGATT™ cell line engineering services to generate a master cell line in a specific cell line-of-choice or to build your cell libraries.*

Advantages of using TARGATT™ Master Cell Lines for gene knockin:

- High efficiency integration
- Site-specific, stable knockin cell line generation
- Single copy gene integration into safe harbor locus
- Gene expression from an active, intergenic locus
- Low off-target integration

Parental Cell Line HEK293 Adherent Cells

Contents

All cell lines and plasmids provided in this kit are sufficient for 9 transfections (i.e., 3 triplicate transfections) according to the given protocol:

- TARGATT™ HEK293 Master Cell line
- TARGATT™ 24 CMV-MCS-attB; Cloning Plasmid
- TARGATT™ 25 mCherry-attB (No Promoter); Positive Control Plasmid

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- TARGATT™ CMV-integrase Plasmid

Quality Control

A certificate of analysis (COA) with detailed quality control information for the cell line and components of the kit will be provided with each shipment.

Shipping

Dry ice

Storage and Stability

Store the TARGATT™ master cell line in liquid nitrogen freezer immediately upon receipt. Store the plasmids at -20°C. Do not freeze-thaw plasmids repeatedly. The products provided in this kit are stable for at least 6 months from the date of receiving when stored as directed.

Safety Precaution

PLEASE READ BEFORE HANDLING ANY FROZEN VIALS. Please wear the appropriate Personal Protective Equipment (lab coat, thermal gloves, safety goggles and a face shield) when handling the cells. Handle the frozen vials with due caution. Please be aware that the following scenario can occur: Liquid nitrogen can leak into the vials when the vials are submerged in liquid nitrogen. Upon thawing, the liquid nitrogen returns to the gas phase, resulting in a dangerous build-up of pressure within the vial. This can result in the vial exploding and expelling not only the vial contents but also the vial cap and plastic fragments of the vial.

Restricted Use

This product is for research use only and not intended for human or animal diagnostic or therapeutic uses.

Schematic Representation of TARGATT™ Knockin Strategy

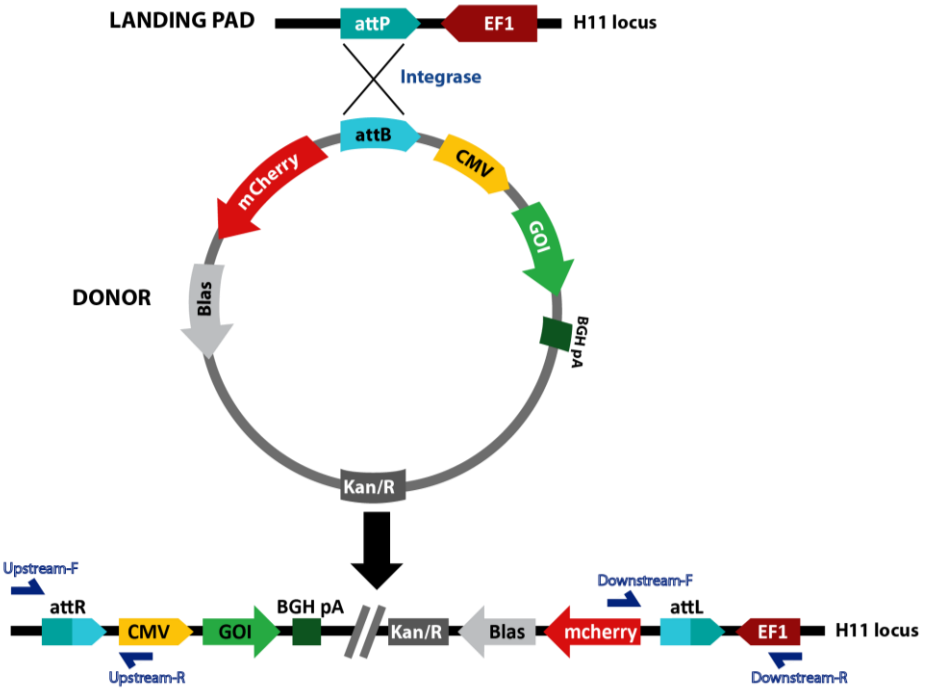

Figure 1. Schematic representation of TARGATT™ site-specific transgene integration mediated by integrase. The TARGATT™ HEK Master Cell Line was engineered with the attP landing pad at the hH11 safe harbor locus. The TARGATT™ plasmid containing the integrase recognition site, attB is used to clone the transgene. The integrase catalyzes an irreversible reaction between the attP site in the genome and attB site in the donor vector, resulting in integration of the gene of interest at the selected H11 locus. The cells containing the gene of interest can be enriched using the selection marker (gray box).

Media and Material

Catalog#	Component	Amount
AST-1305-1	TARGATT™ HEK293 Master Cell Line	1 x 10^6 cells
AST-3064	TARGATT™ 24 CMV-MCS-attB; Cloning Plasmid*#	5 µg
AST-3065	TARGATT™ 25 mCherry-attB (No Promoter); Positive Control Plasmid#†	15 µg
AST-3201	TARGATT™ CMV-integrase Plasmid#†	15 µg

** Amplify the cloning plasmid to clone your gene of interest; # Aliquot into single-use quantities and store at -20°C; do not freeze-thaw the plasmid repeatedly. † The positive control and integrase plasmids are proprietary materials and should not be amplified. The provided quantity is sufficient for 9 transfections (i.e., 3 triplicate transfections). Additional quantities of these plasmids are available for purchase.*

Materials Needed but not Provided

Material	Vendor	Catalog#
Dulbecco's Modified Eagle's Medium (DMEM), High Glucose, No Glutamine	ThermoFisher	11960077
Fetal Bovine Serum (FBS)	Biowest	S1620
100x Glutamax™ Supplement	ThermoFisher	35050061
100mM Sodium Pyruvate	ThermoFisher	11360070
1M HEPES	ThermoFisher	15630080
Gibco™ Penicillin-Streptomycin (10,000 U/mL)	ThermoFisher	15140148
Normocin™ - Antimicrobial Reagent, 500 mg	Invivogen	ant-nr-1
Blasticidin	Invivogen	ant-bl-05
Xfect™ Transfection Reagent	Takara Bio	631318
DPBS, No Calcium, No Magnesium	ThermoFisher	14190136
0.25% Trypsin EDTA (1x)	ThermoFisher	25200056
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich	D2438
NEB® 10-beta Competent E. coli (High Efficiency)	NEB	C3019
Living Colors® pAcGFP1-C1 Vector#	Clontech/Takara	632470
NEB® Hot Start *Taq* DNA Polymerase	NEB	M0495L
Corning® CellBIND® 6-well Clear Multiple Well Plates	Corning	3335
Corning® CoolCell® Freezing System	Corning	UX-04392-

The above reagents are recommended based on our culture protocols. If you are using a similar/ alternate reagent, we recommend that you perform a small-batch test using your preferred reagents. # Any GFP plasmid with size <7kb can be used.

Protocol

1. Preparation of culture medium

1.1 Example preparation of HEK293 medium (complete growth medium)

- 1000 mL DMEM High Glucose, (-) Glutamine, (-) Pyruvate
- 50 ml FBS
- 11 mL 100x GlutaMAX
- 11 mL 100mM Pyruvate
- 27.4 mL 1M HEPES

Notes:

- *The medium should be kept at 4*C. Only aliquots that will be used up that day are warmed up to room temperature or 37*C.*
- *Pen/Strep (Gibco™ 15140) is diluted 200x into the aliquot that will be used. It is *not* added to the stock medium.*
- *Pen/Strep aliquots must be stored at -20*C in a non-cycling freezer (i.e. freezers that build-up ice). Aliquots are only thawed once.*

1.2 Freezing medium

- 90% HEK293 medium
- 10% DMSO

1.3 Example preparation of sorting medium: (for FACS recovery)

- 10.00 mL HEK293 medium
- 0.53 mL FBS
- 53 µL Pen/Strep
- 22 µL Normocin

2. Thawing and culturing cryopreserved cells

To ensure the highest level of viability, thaw the vial and initiate the culture as soon as possible upon receipt. If upon arrival, continued storage of the frozen culture is necessary, it should be stored in liquid nitrogen vapor phase and not at -70°C. Storage at -70°C will result in loss of viability.

2.1 Thaw the vial containing HEK293 cells by gentle agitation in a 37°C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (approximately 2 minutes).
2.2 Remove the vial from the water bath as soon as the contents are thawed, and decontaminate by dipping in or spraying with 70% ethanol.
Note: All of the operations from this point on should be carried out under strict aseptic conditions.
2.3 Transfer the contents of the vial equally into 2 wells of a 6-well plate (1/2 vial in each well; 2-fold dilution).
2.4 Add “enriched” complete growth medium (complete growth medium in step 1.1 but with 9% FBS) while moving the plate in a figure-8 pattern several times.
2.5 Incubate the culture in a suitable incubator at 37°C in 5% CO_2 for 24 hours.
2.6 After 24 hours, change media to complete growth medium (step 1.1).
2.7 Incubate the culture at 37°C in 5% CO_2.

3. Sub-culturing procedure (maintenance)

Volumes are given for a 10 cm^2 well in a multi-well plate (6-well plate). Corning® CellBIND® 6-well Clear Multiple Well Plates or poly-lysine coated plates are highly recommended for subculturing this product. Increase or decrease the amount of dissociation medium needed proportionally for culture vessels of other sizes.

3.1 Remove and discard the spent culture medium.
3.2 Add 750 µL of 4-fold diluted 0.25% Trypsin-EDTA (T/4) solution (diluted with DPBS) to one well and observe cells under an inverted microscope until the cell layer is dispersed (usually 3-5 minutes).
Note: To avoid clumping do not agitate the cells by hitting or shaking the plate while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.
3.3 Add 750 µL of complete growth medium and swirl to mix. Then break-up the cell clumps by gently pipetting.
3.4 Add appropriate aliquots of the cell suspension to new culture vessels.
3.5 Incubate cultures in a 37°C/ 5% CO_2 incubator.
3.6 Subculture before cell confluency reaches 100%.

- Subcultivation ratio: 1:6 to 1:20 weekly, depending on timing of expected usage
- Medium renewal: Every 2 to 3 days

4. Cryopreserving cells

We recommend cryopreserving the cells from a 70-90% confluent well of a 6-well plate (i.e. 10 cm^2 growth area) in 1 mL of freezing medium per cryogenic vial.

4.1 Remove the medium (supernatant) from culture well or flask.
4.2 Add adequate amount of freezing media to cells (1 mL for 10 cm^2 growth area), and detach the cells by pipetting.
4.3 Transfer approximately 1 to 1.5 mL of the cell suspension to a labeled cryogenic vial.
4.4 Move the vial(s) to a Corning® CoolCell® Freezing System for cryogenic vials and move the CoolCell® holder to a -80*C freezer.
Note: Try to place the vials symmetrically in CoolCell® holder to facilitate even freezing across all vials.
4.5 After one day, move the cryogenic vials to liquid nitrogen storage.
Note: Place the cryogenic vials on dry-ice while transferring from -80°C freezer to the liquid nitrogen tank.

5. Transfection procedure

5.1 Plasmid amplification and cloning with TARGATT™ 24 CMV-MCS-attB cloning plasmid

5.1.1 Amplify the TARGATT™ 24 CMV-MCS-attB Cloning Plasmid using NEB® 10-beta Competent E. coli (High Efficiency) competent cells
Note: Do not use 5-alpha competent cells to transform the plasmids.
5.1.2 Aliquot into single-use quantity and store at -20°C. Do not freeze-thaw the plasmid repeatedly. We recommend using 2-5 µg of the plasmid for each transfection
5.1.3 Clone the gene of interest into the TARGATT™ 24 CMV-MCS-attB Cloning Plasmid to make the donor plasmid.

5.2 Seeding for transfection the next day in a 24-well plate

5.2.1 Warm up the following reagents at room temperature:
- Aliquot of HEK293 medium without Pen/Strep
- Pen/Strep
- Trypsin (T/4) = 0.25% trypsin 4-fold diluted with DPBS
- DPBS (for resuspending cells after centrifugation)

5.2.2 Aspirate the medium (supernatant) from the culture vessel.
5.2.3 Immediately add 750 µL T/4 solution per well of a 6-well plate.
5.2.4 Allow the trypsinization to work for ~2-5 minutes.
5.2.5 Add 750 µL complete growth medium and swirl to mix. Then break-up the cell clumps by pipetting.
5.2.6 Transfer the entire content of the well to a labeled 1.5 mL centrifuge tube.
5.2.7 Centrifuge at 200 x g for 5 minutes.
5.2.8 Aspirate most of the supernatant, leaving a little liquid at bottom. Using a pipette remove rest of supernatant with pipette without disturbing cell pellet.
5.2.9 Resuspend the cell pellet in 1 mL DPBS and mix by pipetting a couple of times.
Note: At this point, combine resuspended cells from multiple wells if necessary in a 15 mL tube for counting.
5.2.10 Count cells using trypan blue and an automated cell counter.
5.2.11 See the cells in no-antibiotic growth medium such that their concentration will be 1 x 10^5 cell/mL.
5.2.12 Pipet 0.5 mL of the cell solution into each desired well of the 24-well plate (i.e. 50,000 live cells per 2-cm^2 well).
5.2.13 Distribute the cells evenly by quickly moving the plate in a figure-8 pattern several times.
5.2.14 Incubate at 37°C in 5% CO_2.

5.3 Transfection using Xfect™ Transfection Reagent

5.3.1 Warm up the following reagents at room temperature:
- Xfect™ Transfection Reagent (stored at 4°C)
- Xfect™ Transfection Buffer (stored at 4°C)

5.3.2 Calculate the volume of the Xfect™ transfection reagent, buffer and plasmid volume to be mixed.
Note: The Xfect™ to plasmid ratio is 0.3 µL Xfect™ per 1 µg plasmid. However, do not pipet less than 1µL (i.e. always prepare at least a 3.33 µg plasmid mix, even if only transfecting 2 µg, 1 µg, etc).

Table 1. Example transfection mix for transfecting 2 µg GFP plasmid into three wells of a 24-well plate for reagent validation[#]

Reagent	Amount
GFP Plasmid*	6.6 µg**
Xfect™ transfection reagent	1.98 µL
Xfect™ buffer	q.s.
Final volume	**109.9 µL**

** Any GFP plasmid < 7kb can be used. ** Amount of plasmid DNA to be used is given in µg quantity. Calculate the appropriate volume based on the concentration of the plasmid. [#] Amount of transfection mix recommended for pipetting 33.3 µL in three wells.*

5.3.3 For integrase-mediated integration transfections, we recommend a 3:1 donor:integrase mass ratio (i.e. three-fold more of the donor by mass).

Table 2. Transfection of donor or positive control plasmid AST-3065 (mCherry-attB)[#]

Reagent	Amount
Integrase plasmid	1.65 µg**
Donor or positive control plasmid	4.95 µg**
Xfect™ transfection reagent	1.98 µL
Xfect™ buffer	q.s.
Final volume	**109.9 µL**

*** Amount of plasmid DNA to be used is given in µg quantity. Calculate the appropriate volume based on the concentration of the plasmid. [#] Amount of transfection mix recommended for pipetting 33.3 µL in three wells.*

5.3.4 Label 1.5 mL microcentrifuge tubes.
5.3.5 If the medium that the cells are in has Pen/Strep, carefully replace it with warm non-Pen/Strep medium.
5.3.6 Add calculated amount of Xfect™ buffer and plasmid to the microcentrifuge tube.
5.3.7 Vortex or pipet the Xfect™ reagent to fully resuspend it, and then transfer the calculated amount of Xfect™ into the plasmid solution.
5.3.8 Quickly vortex the transfection solution to mix it well. Allow it to incubate at room temperature for at least 10 minutes (but no longer than 15 minutes).
5.3.9 Transfer the appropriate amount of mix to each well of the culture plate without disturbing the attached cells. (For example, to transfect 2 µg plasmid DNA, pipet 33.3 µL of the transfection mix into each desired well of the 24-well plate).
Note: Give a quick shake immediately after transferring the transfection mix to each well.
5.3.10 Incubate at 37*C/ 5% CO_2 for 72 hours. We have observed the highest integration efficiencies ~72 hours after transfection (no medium change is required).
5.3.11 After the 72-hour incubation, cells are ready to be enriched (or sorted, if required).
Note: If sorting, please sort the cells into the sorting medium (step 1.3) for robust recovery of cells.

5.4 Enrichment using Blasticidin selection

5.4.1 Seventy-two (72) hours after transfection, expand the cells 20-fold. (Ex. from one well of a 24-well plate (2 cm^2) into four wells of a 6-well plate (40 cm^2). Use trypsin so that clumps are broken up into single cells (to prevent negative cells from being shielded from blasticidin).

5.4.2 After 24 hours, replace the growth medium with blasticidin selection medium (= growth medium plus 10 ug/mL blasticidin).

5.4.3 Maintain selection pressure for 2 weeks, or until the desired level of enrichment is reached (can be monitored by checking for mCherry fluorescence using a microscope).

5.4.4 Before the 2 weeks have been reached, the cells should be split 20-fold every time they reach 80-90% confluence.

5.4.5 For a 20-fold split using a 6-well plate:

- Remove medium from the well.
- Add 750 µL of 0.0625% trypsin (4-fold dilution of 0.25%) to the well.
- Incubate for 2-5 minutes until the cells start to detach.
- Add 750 µL of growth medium to neutralize the trypsin and break-up the cells by pipetting.
- Transfer 75 µL of the suspended cells into a new well of a 6-well plate with 2.5 mL blasticidin selection medium.

Supporting Data

Confirmation of site-specific CMV-MCS plasmid integration

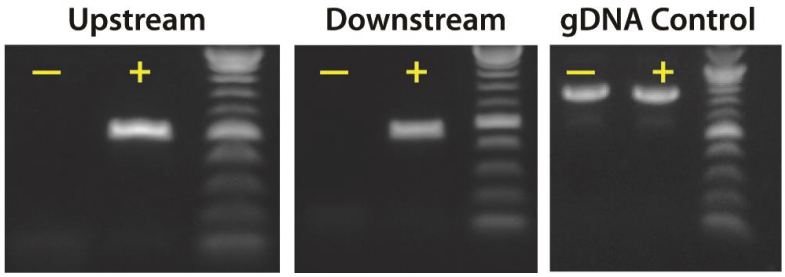

Figure 2. PCR gel electrophoresis to confirm the knockin of TARGATT™ 24 CMV-MCS-attB plasmid mediated by the TARGATT™ Integrase plasmid, after transfection into the TARGATT™ HEK293 Master Cell Line. Two sets of primers were used to confirm knockin: Upstream (512 bp) and Downstream primers (464 bp). The Human control primers (777 bp) was also used as a control to check the integrity of the cells and the genomic DNA (gDNA). Negative control (-) represents cells transfected with the TARGATT™ 24 CMV-MCS-attB plasmid and a mutant TARGATT™ Integrase Plasmid that is deficient for integration. DNA ladder used was NEB's 1 kb Plus DNA Ladder. Refer to appendix section for primer sequence and PCR protocols.

mCherry expression 72 hours post-transfection

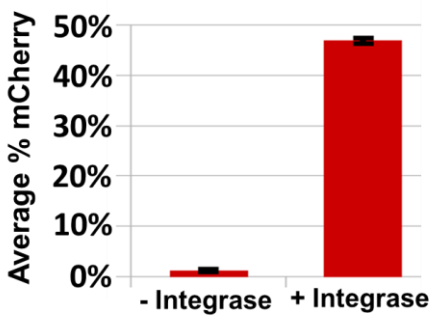

Figure 3. The mCherry integration into the TARGATT™ HEK293 master cell line mediated by the integrase, 72 hours post-transfection. Cells were transfected with the mCherry positive control plasmid and either the provided TARGATT™ integrase plasmid (+Integrase) or a mutant TARGATT™ integrase plasmid deficient for integration (-Integrase). The mCherry plasmid has no promoter and requires the ubiquitous EF1 promoter in the landing pad after integration to express the reporter gene. The integration efficiency of mCherry knockin into landing pad was >40%, without selection. Data represents the mean ± SE of two representative experiments done in triplicates.

mCherry expression after Blasticidin enrichment

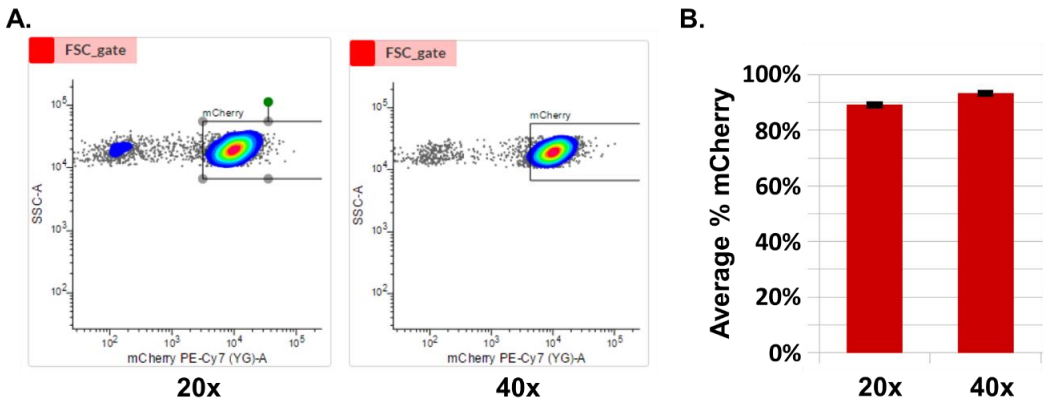

Figure 4. Blasticidin enrichment of TARGATT™ HEK293 cells with a knocked-in mCherry-blasticidin plasmid. Cell pools (with 20x and 40x split ratio) were enriched in selection medium for 3 weeks (without cell sorting). (A Flow-cytometric analysis of mCherry expression after enrichment. (B) Summary of enrichment results for two representative experiments done in triplicates (N=3). Data shown is mean ± SE. The enrichment of mCherry was about 90% after blasticidin selection.

Reference

- Chi, X., Zheng, Q., Jiang, R., Chen-Tsai, R. Y., & Kong, L. J. (2019). A system for site-specific integration of transgenes in mammalian cells. *PLOS ONE*, *14*(7), e0219842.

Appendix

The Touchdown PCR method was used to perform the PCR reactions defined in Figure 2 (Supporting Data). Touchdown PCR refers to a type of PCR technique in which the initial annealing temperature is set higher than the calculated primer melting temperature (Tm) and is gradually decreased until the calculated Tm is reached. The touchdown PCR increases specificity of primer annealing and amplification of the desired amplicons. In the touchdown PCR protocol provided below, the starting annealing temperature is set to Tm + 5°C and reduced by 0.5°C per cycle for 10 cycles.

(i) Set up PCR reaction on ice according to manufacturer's instruction for the *Taq* polymerase being used. Below is an example for PCR solution using the NEB® Hot Start *Taq* polymerase:

Component	Amount
Nuclease-free water	40.50 µL
10X NEB Standard *Taq* Buffer	5.00 µL
10 mM dNTP (NEB)	1.00 µL
10µM Forward Primer	1.00 µL
10µM Reverse Primer	1.00 µL
gDNA	1.00 µL
NEB Hot Start *Taq* Polymerase	0.50 µL
Total Volume	**50.00 µL**

(ii) Primer sets: Please see Figure 1 (schematic) for location of primers

Upstream Primers:	Forward (F): 5'-GTTGTGTGTTTGGTTGTGTGGTGG-3' Reverse (R): 5'-AATCCCCGTGAGTCAAACCG-3'

Downstream Primers	Forward (F): 5'-CCTTGTAGATGAACTCGCCGT-3' Reverse (R): 5'-GGTGTCGTGATTATTCGAAGGG-3'
Human Control Primers	Forward (F): 5'-ACCTCCAGTTAGGAAAGGGGACT-3' Reverse (R): 5'-AAGTTTTTCTTGAAAACCCATGGAA-3'

(iii) Perform PCR amplification using the following programs for each primer sets.

Step	# of Cycles	Upstream		Downstream		Human Control	
		Temp	Time	Temp	Time	Temp	Time
1	1	95°C	30 s	95°C	30 s	95°C	30 s
2 (Touchdown: decrease 0.5°C per cycle)	10	95°C 63°C 72°C	15 s 15 s 31 s	95°C 62°C 72°C	15 s 15 s 29 s	95°C 62°C 72°C	15 s 15 s 55 s
3 (Main PCR)	30	95°C 58°C 72°C	15 s 15 s 31 s	95°C 57°C 72°C	15 s 15 s 29 s	95°C 57°C 72°C	15 s 15 s 55 s
4	1	72°C	5 min	72°C	5 min	72°C	5 min
5		4°C	Hold	4°C	Hold	4°C	Hold